

## Isozyme Variation in Salamanders of the *nebulosus-lichenatus* Complex of the Genus *Hynobius* from Eastern Honshu, Japan, with a Description of a New Species

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**Abstract:** Isozyme variation was studied in 244 individuals of the *Hynobius nebulosus-lichenatus* complex from eastern Honshu, between the Kinki and Tohoku Districts, Japan. Starch gel electrophoresis was used to study variation at 17 loci. Six named species (*H. lichenatus*, *H. nigrescens*, *H. takedai*, *H. abei*, *H. tokyoensis*, *H. nebulosus*) were clearly separated but by relatively low genetic distances (minimum value of Nei's  $D=0.22$ ), although genetic relationships among these species were largely in disaccord with the currently recognized phylogenetic relationships. The genetic distances between the population from Hakuba and all the named species of this complex proved to be sufficiently high to differentiate the population at a specific rank. On the basis of this result, the population from Hakuba is described as a new species, *Hynobius hidamontanus*. This species is distributed in the montane region of Nagano Prefecture, in the Hida Mountains of the Chubu District, Central Japan. It belongs to the *nebulosus-lichenatus* complex of *Hynobius*, and is characterized by a shallow vomerine teeth series, small number of vomerine teeth, intermediate body shape between *H. lichenatus* and *H. nebulosus*, absence of striations on the egg sac envelope, and unique electrophoretic pattern of isozymes.

**Key words:** Caudata; *Hynobius hidamontanus*; Central Japan; Electrophoresis; Taxonomy

In 1980, we reported the occurrence of a unique salamander with the characteristics similar to *H. lichenatus* from Hakuba-mura, in the northwestern part of Nagano Prefecture (Matsui and Matsui, 1980). Subsequent surveys both for the population from Hakuba and for many populations of *H. lichenatus* further proved our original assumption (Matsui and Matsui, 1980) that the salamander from Hakuba could be constantly distinguished from *H. lichenatus* in several characteristics, especially in the structure of the egg sac. However, as is the case for most Japanese salamanders (Matsui and Miyazaki, 1984), the salamander in question was sometimes morphologically similar enough to other primarily lentic breeding *Hynobius* species (*nebulosus* and *lichenatus* groups of Sato, 1943), that its distinct specific status could be questioned.

Meanwhile, Nambu (1983) ascertained our earlier assumption that the *lichenatus*-like population from O-umi, Niigata Prefecture, was different from typical *lichenatus* in the absence of striations on the egg sac. In addition, he reported the occurrence of a similar salamander from Ooyama, Toyama Prefecture. Both of these localities are geographically close to Hakuba, surrounding the northern half of

the Hida Mountains. Further, populations of wide-ranging *H. lichenatus*, which is seemingly closest to these salamanders, have been shown to be morphologically very heterogeneous (Hasumi and Iwasawa, 1987).

Isozyme analysis has proven to be a highly useful technique in the taxonomy of various lineages in urodele amphibians (e.g., Hedgecock and Ayala, 1974; Wake et al., 1978; Hanken and Wake, 1982), and this also holds for Japanese newts (Hayashi and Matsui, 1986). However, in the Japanese salamanders, the relationship between genotypic and phenotypic evolution remains poorly understood. Moreover, isozymic divergence might not be coupled with morphological evolution and might permit an independent assessment of phylogenetic relationships among Japanese salamanders.

I, therefore, am trying to examine isozymic variation in the Japanese salamanders using starch gel electrophoresis. This paper presents the results of a study of isozyme variation of 17 loci in primarily lentic breeding *Hynobius* species from Honshu from the Kinki District northwards. In addition, on the basis of the results of this analysis, I describe the salamander from Hakuba as a new species.

### MATERIALS AND METHODS

*Electrophoresis.*—I tried to obtain samples

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from throughout the collective range of the *nebulosus-lichenatus* complex around Hakubamura (Fig. 1). The isozymes of 244 specimens were examined electrophoretically as shown in Table 1. Populations from Hakuba and O-umi (Pops. 1, 2) are morphologically similar to each other, and tentatively treated as a single taxon in the analysis. Population 19 is identical to the salamander which Nambu (1986) assigned to *H. takedai*.

In the laboratory, the tissues of liver, abdominal muscle, and spleen were removed and stored at  $-84^{\circ}\text{C}$  until use. Voucher specimens were fixed in 10% formalin, preserved in 70% ethanol, and deposited in Matsui's collection at Kyoto University, except for 15 specimens of Pop. 1 (Hakuba) and two specimens of Pop. 2 (O-umi). The former 15 specimens are in the collection of the Osaka Museum of Natural History (OMNH) and the National Science Museum, Tokyo (NSMT), while only tissue samples of the latter two specimens were provided by Mr. Nambu of the Toyama Science Museum. Supernate fractions of the homogenates were run on standard horizontal gels of starch, containing Connaught starch at a concentration of 11.5%. Ten enzyme systems encoded by 17 presumptive gene loci were resolved for all taxa (Table 2). Tris citrate buffer pH 7.0 (1 : 15 dilution of electrode buffer for gel) was used throughout the analysis. Staining protocols are from Ayala et al. (1972), Harris and Hopkinson (1976), or Sato (1982). Multiple loci were alphabetically numbered from cathode to anode, and electromorphs were assigned letters according to their mobility, beginning with the electromorph closest to the cathode.

Individual genotypes were used to calculate allele frequencies for all taxa, and these in turn were used to calculate matrices of genetic identity and distance (Nei, 1978; Rogers, 1972). The distance coefficients were then clustered by the unweighted pair group method (UPGMA: Sneath and Sokal, 1973) to provide a general estimate of the overall genetic relationships among the taxa.

**Morphological comparisons.**—For morphological comparisons with the population from Hakuba, five named species were used: *H. lichenatus* from Aomori (near type locality and northernmost limit of species distribution) and Tochigi (southernmost limit), *H. takedai* from Ishikawa (near type locality), *H. abei* from Kyoto (near type locality), *H. tokyoensis* from Chiba, and *H. nebulosus* from Shiga and

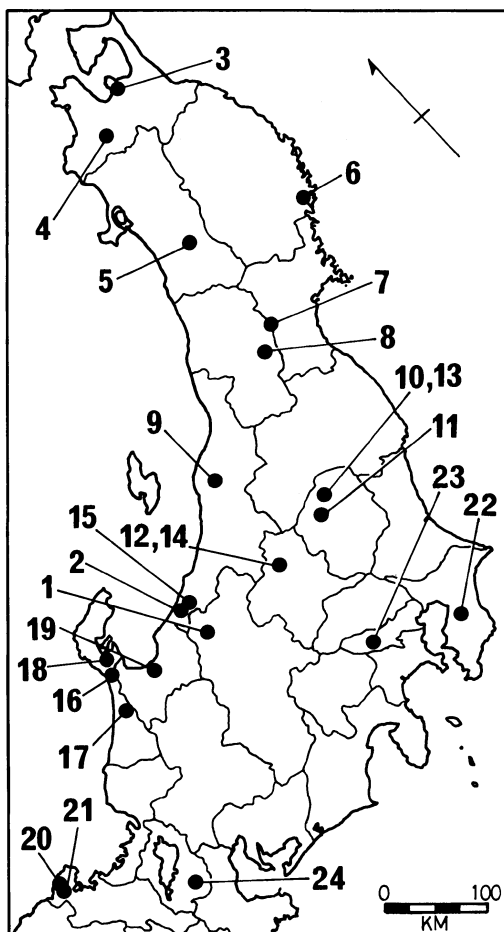


FIG. 1. Map of Honshu from the Kinki District northwards, showing the sampling localities of the *Hynobius nebulosus-lichenatus* complex used in the electrophoretic analysis. Numbers refer to those listed in Table 1.

Nagasaki (near type locality). The number of specimens varied among characters, and the maximum total was 289. All measurements were taken on preserved specimens. The following eight measurements were taken: 1) snout-vent length (SVL), from snout to anterior angle of cloaca = sum of 2) and 3); 2) head length (HL), from tip of snout to gular fold; 3) trunk length (TRL), from gular fold to anterior angle of cloaca; 4) tail length (TAL), from anterior angle of cloaca to tip of tail; 5) head width (HW), measured at jaw articulation; 6) maximum tail height (MTAH); 7) length of vomerine teeth series (TVL); and 8) width of vomerine teeth series (VTW). The number of costal grooves was counted, including the

TABLE 1. Localities and size of samples used for electrophoretic analysis.

Species	Pop. no.	Locality	N
<i>Hynobius</i> sp.	1	Hakuba-mura, Nagano Pref.	25
	2	O-umi-machi, Niigata Pref.	2
<i>H. lichenatus</i>	3	Aomori-shi, Aomori Pref.	11
	4	Hirosaki-shi, Aomori Pref.	9
	5	Omagari-shi, Akita Pref.	7
	6	Sanriku-cho, Iwate Pref.	2
	7	Funagata-machi, Yamagata Pref.	4
	8	Yamagata-shi, Yamagata Pref.	11
	9	Tagami-machi, Niigata Pref.	11
	10	Shiobara-machi, Tochigi Pref.	10
	11	Fujiwara-machi, Tochigi Pref.	2
	12	Minakami-machi, Gunma Pref.	28
<i>H. nigrescens</i>	13	Shiobara-machi, Tochigi Pref.	2
	14	Minakami-machi, Gunma Pref.	3
	15	Itoigawa-shi, Niigata Pref.	10
	16	Hakui-shi, Ishikawa Pref.	3
	17	Kanazawa-shi, Ishikawa Pref.	3
<i>H. takedai</i>	18	Tatsuruhama-machi, Ishikawa Pref.	14
	19	Toyama-shi, Toyama Pref.	5
<i>H. abei</i>	20	Amino-cho, Kyoto Pref.	18
	21	Mineyama-cho, Kyoto Pref.	4
<i>H. tokyoensis</i>	22	Nagara-machi, Chiba Pref.	23
	23	Itsukaichi-machi, Tokyo Pref.	9
<i>H. nebulosus</i>	24	Hino-cho, Shiga Pref.	28

TABLE 2. Loci examined and Enzyme Commission (E.C.) numbers.

Enzyme	Locus	E.C. no.
Acid phosphatase	Acp-A	3.1.3.2
Acid phosphatase	Acp-B	3.1.3.2
Aspartate aminotransferase	M-Aat-A	2.6.1.1
Aspartate aminotransferase	S-Aat-A	2.6.1.1
Esterase	Est-1	3.1.1.1
Esterase	Est-2	3.1.1.1
Glucosephosphate isomerase	Gpi-A	5.3.1.9
L-Lactate dehydrogenase	Ldh-A	1.1.1.27
Malate dehydrogenase	M-Mdh-A	1.1.1.37
Malate dehydrogenase	S-Mdh-A	1.1.1.37
Malic enzyme	M-Me-A	1.1.1.40
Malic enzyme	S-Me-A	1.1.1.40
Phosphoglucomutase	Pgm-A	5.4.2.2
Phosphoglucomutase	Pgm-B	5.4.2.2
Phosphoglucomutase	Pgm-C	5.4.2.2
6-Phosphogluconate dehydrogenase	Pgdh-A	1.1.1.44
Superoxide dismutase	S-Sod-A	1.15.1.1

axillary groove. Overlap of finger and toe tips when both limbs were adressed to the body was recorded by the number of costal folds between the tips, and consequently, 'plus' indicates overlap and 'minus' indicates separation. For morphometric comparisons, Student's t-test and Aspin-Welch's t-test were used. A two-tailed Mann-Whitney's U test was performed for ratio values, and chi-square or Fisher's exact tests were run to detect the presence or absence of differences in the fre-

quency distributions. The significance level was 95% in all these statistical tests.

#### RESULTS

Of the 17 loci scored (Table 3), two (M-Aat-A, Acp-B) showed no variation in all populations, and the remaining 15 were polymorphic in at least some populations. The frequency of polymorphic loci in the 24 populations ranged from 5.9% (Pops. 6 and 21) to 64.7% (Pop. 9) (mean=19.9%). The mean

number of alleles per locus per sample ranged from 1.06 (Pops. 6, 21) to 1.88 (Pop. 24). In S-Sod-A, Gpi-A, Pgm-C, and M-Me-A, there were electromorphs unique to single populations. Fixed differences were identified among most of the species, but completely fixed differences were not found between *H. lichenatus* and *H. tokyoensis* or *H. nigrescens* and *H. nebulosus*. Combined populations of Hakuba and O-umi (Pops. 1, 2) had fixed differences between the six named species at least at one locus. *H. abei* was most distinct, with electromorphs unique to the species in five loci.

As shown in Table 4, the mean of Nei's (1978) distances among conspecific populations of five named species was large in the wide-ranging species—0.152 in *H. lichenatus* (Pops. 3–12) and 0.081 in *H. nigrescens* (Pops. 13–17), but was small in the species with a more limited distribution range—0.026 in *H. takedai* (Pops. 18, 19), 0.055 in *H. tokyoensis* (Pops. 22, 23), and 0.036 in *H. abei* (Pops. 20, 21). The ground mean for these values was 0.070. Interspecies distances were always larger than intraspecies ones, and the mean distances among the six named species plus combined populations from Hakuba and O-umi varied from 0.223 (*H. lichenatus*–*H. tokyoensis*) to 0.919 (*H. takedai*–*H. abei*), with an overall mean of  $0.476 \pm 0.189$  (SD). These tendencies were nearly identical in the Rogers' (1972) distances (Table 4).

The results of UPGMA clustering were essentially identical for the two distance matrices (Nei, 1978; Rogers, 1972), and only the phenogram from Nei (1978) distances is presented (Fig. 2) and discussed. As shown in Fig. 2, the first major dichotomy clearly separates *H. abei* from the others. The second major dichotomy separates two clusters. In the first cluster, one subcluster contains *Hynobius* sp. from Hakuba and O-umi, and another the groups *H. takedai*, *H. nigrescens*, and *H. nebulosus*. In the second subcluster, *H. takedai* is separated from *H. nigrescens* and *H. nebulosus*. In the second cluster, one subcluster contains *H. lichenatus* and the other *H. tokyoensis*.

In this way, the phenogram derived from isozyme variation coincided well with the current classification, and no population of one species was grouped with other species in the phenogram. The six named species treated in the present study were differentiated from each other at a mean D value greater than 0.22.

In this regard, the salamanders from Hakuba

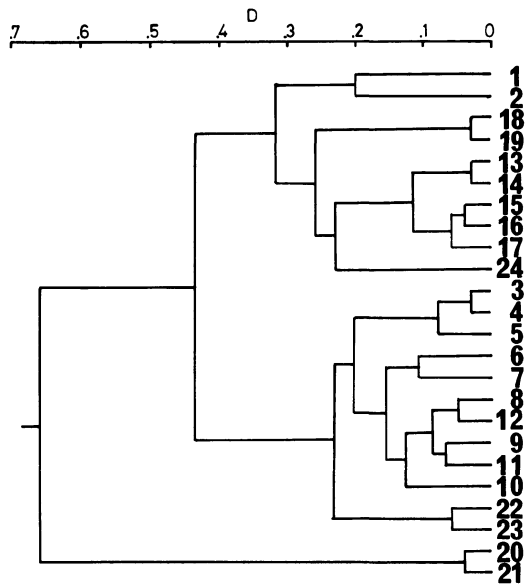


FIG. 2. Phenogram based on Nei's (1978) unbiased genetic distances and using the UPGMA algorithm. Figures refer to the population numbers listed in table 1.

and O-umi (Pops. 1 and 2) are remarkable; when these two populations are combined, they differed from the closest cluster (*H. nigrescens*, *H. takedai* and *H. nebulosus*) at a mean value of D as large as 0.312. This value can be regarded as large enough to specifically separate these two populations from all other named species. The populations from Hakuba and O-umi themselves, however, are also different from each other at a D of 0.195. Unfortunately, the small sample size of the O-umi population prohibits further discussion, and it is at present unknown whether this value suggests further separation within these two populations, or is merely a result of biases due to the small sample size of the O-umi population. Thus, it seems at present best to define the taxonomic status of the Hakuba population alone, leaving the O-umi population for future study. The Hakuba population constantly showed fixed differences from each of the other named species, although no electromorphs unique to the population were detected. The mean Nei distances between the Hakuba population and the other named salamander species are: 0.363 relative to *H. lichenatus*, 0.279 to *H. nigrescens*, 0.384 to *H. takedai*, 0.615 to *H. abei*, 0.392 to *H. tokyoensis*, and 0.298 to *H. nebulosus*. These values ( $>0.22$ ), when compared with those



TABLE 3. Continued.

Locus	<i>H. nigrescens</i>					<i>H. takadai</i>			<i>H. abei</i>		<i>H. tokyoensis</i>			<i>H. nebulosus</i>
	Pop. 13	Pop. 14	Pop. 15	Pop. 16	Pop. 17	Pop. 18	Pop. 19	Pop. 20	Pop. 21	Pop. 22	Pop. 23	Pop. 24		
Acp-A	b	b	b(900) c(100)	b	b	b(036) c(964)	c	b	b	b	b	a(089) b(893) c(018)		
Acp-B	a	a	a	a	a	a	a	a	a	a	a	a		
M-Aat-A	a	a	a	a	a	a	a	a	a	a	a	a		
S-Aat-A	c(250) d(750)	d	c(650) d(350)	b(167) c(833)	b(167) c(667) d(167)	c(964) d(036)	c	d	d	b	b	a(143) c(804) d(054)		
Est-1	a(750) b(250)	a	a	a	a	a	a	a(972) b(028)	a	a(435) b(565)	a	c		
Est-2	c	c	b(700) c(300)	b(333) c(667)	b	b	a(200) b(800)	c	c	a(043) b(957)	b	a(732) b(268)		
Gpi-A	c	c	c	c	c	c	c	b	b	b(978) c(022)	b(833) c(167)	a(232) b(321) c(411) d(036)		
Ldh-A	b	b	b	b	b	b(929) c(071)	b	a(944) b(056) d(222)	a	c	c	b		
M-Mdh-A	b	b	b(600) d(400)	b	b	a	a	d(222) e(778)	e	b	b	b		
S-Mdh-A	b	b	b(800) c(200)	b	b	b(929) c(071)	b	d(722) d(278)	d	a(978) b(022)	a	b(982) c(018)		
M-Me-A	b	b	b(750) c(250)	b	b	b(929) c(071)	b	b	b	b(826) c(174)	b(333) d(333)	b(893) c(107)		
S-Me-A	c	c	c	c	c(667) d(333)	a(071) b(143) c(786)	b(700) c(100) d(200)	c	c	c	a(556) c(444)	b(036) c(964)		
Pgm-A	b	a(667) b(333)	a(850) b(150)	a	a	a	a	b(972) c(028)	b(750) c(250)	a	a	a(982) b(108)		
Pgm-B	a	a	a	a(667) b(333)	a(833) b(167)	a	a	a	a	a	a	a		
Pgm-C	b	a(167) b(833)	b	b(833) c(167)	a(667) b(333)	b	b	b	b	b	b	b(732) c(268)		
Pgdh-A	c	c	c	c	c	c	c	b	b	c	b(222) c(778)	c		
S-Sod-A	c	c	c	c	c	c	c	a	a	c(978) d(022)	c(889) d(111)	b(786) c(179) d(036)		

TABLE 4. Matrices of Nei's (1978) genetic distance (above diagonal) and Rogers' (1972) genetic distance (below diagonal) for populations of the *Hynobius nebulosus-lichenatus* complex studied.

Population	Population											
	1	2	3	4	5	6	7	8	9	10	11	12
1		.195	.253	.256	.214	.463	.406	.321	.378	.416	.487	.435
2	.215		.316	.357	.302	.564	.526	.459	.374	.513	.661	.587
3	.264	.337		.026	.060	.197	.184	.200	.184	.249	.313	.259
4	.254	.333	.105		.074	.134	.179	.163	.194	.234	.274	.214
5	.250	.344	.154	.169		.182	.146	.122	.135	.148	.271	.181
6	.385	.441	.244	.164	.242		.100	.091	.169	.237	.207	.130
7	.383	.456	.225	.255	.220	.192		.072	.109	.179	.141	.170
8	.309	.426	.250	.213	.187	.143	.158		.052	.121	.069	.042
9	.399	.407	.258	.266	.217	.249	.220	.156		.132	.066	.084
10	.394	.476	.306	.278	.219	.283	.262	.195	.238		.152	.081
11	.412	.529	.329	.295	.304	.227	.226	.135	.193	.212		.104
12	.362	.478	.272	.228	.226	.146	.226	.090	.187	.148	.138	
13	.353	.324	.258	.300	.320	.391	.369	.395	.386	.436	.442	.414
14	.309	.280	.219	.253	.293	.333	.338	.343	.353	.415	.404	.364
15	.278	.324	.231	.282	.326	.406	.401	.401	.383	.452	.429	.418
16	.235	.219	.184	.185	.264	.309	.380	.347	.320	.414	.414	.365
17	.216	.267	.241	.253	.314	.339	.423	.402	.395	.447	.488	.415
18	.316	.393	.301	.358	.431	.506	.507	.478	.448	.492	.493	.470
19	.352	.342	.327	.392	.464	.543	.544	.515	.489	.520	.535	.506
20	.459	.545	.444	.444	.474	.488	.409	.430	.497	.564	.458	.507
21	.467	.554	.457	.452	.486	.494	.420	.437	.507	.574	.465	.516
22	.341	.550	.279	.251	.285	.193	.198	.205	.303	.273	.230	.218
23	.364	.519	.316	.301	.332	.243	.262	.230	.331	.333	.236	.269
24	.310	.407	.284	.327	.314	.462	.417	.443	.425	.444	.514	.462

TABLE 4. Continued.

	Population											
	13	14	15	16	17	18	19	20	21	22	23	24
.403	.348	.242	.212	.191	.353	.414	.605	.625	.388	.396	.298	
.309	.285	.291	.182	.241	.433	.362	.734	.762	.732	.654	.422	
.216	.174	.143	.108	.174	.274	.324	.542	.563	.247	.285	.254	
.280	.232	.216	.151	.229	.391	.448	.546	.565	.218	.259	.309	
.309	.257	.275	.198	.285	.472	.534	.585	.609	.254	.308	.277	
.456	.359	.437	.332	.353	.666	.759	.647	.660	.165	.200	.559	
.366	.351	.407	.353	.438	.601	.695	.451	.475	.115	.184	.428	
.436	.359	.414	.338	.438	.604	.692	.512	.526	.148	.165	.524	
.368	.310	.338	.260	.382	.480	.560	.592	.610	.229	.246	.466	
.483	.443	.489	.409	.506	.627	.660	.777	.799	.234	.330	.493	
.537	.464	.498	.453	.590	.646	.738	.568	.582	.214	.205	.662	
.504	.422	.475	.394	.489	.607	.689	.701	.715	.205	.242	.565	
	.025	.104	.101	.185	.322	.370	.421	.463	.439	.500	.290	
.078		.074	.066	.125	.282	.330	.456	.483	.398	.428	.265	
.185	.156		.030	.053	.119	.173	.556	.607	.309	.317	.200	
.163	.123	.134		.045	.168	.212	.646	.667	.327	.352	.176	
.232	.182	.161	.114		.165	.198	.791	.812	.321	.326	.210	
.314	.284	.180	.217	.209		.026	.849	.878	.464	.471	.335	
.337	.308	.224	.240	.233	.066		.964	.986	.562	.543	.371	
.368	.388	.466	.501	.570	.579	.617		.036	.665	.683	.775	
.397	.397	.485	.510	.578	.590	.626	.074		.681	.696	.806	
.373	.364	.314	.335	.335	.395	.452	.498	.507		.055	.400	
.438	.400	.344	.371	.343	.409	.449	.519	.525	.122		.493	
.309	.288	.262	.228	.267	.335	.362	.556	.568	.370	.439		

observed among the named salamander species, seem to be sufficiently large for assigning the population to an undescribed distinct species. Although less clearly, the morphological and ecological evidence reported previously (Matsui and Matsui, 1980) also indicates its distinct taxonomic status. Therefore, I herewith describe the population from Hakuba as:

*Hynobius hidamontanus*

M. Matsui sp. nov.

[Japanese name: Hakuba-sansyōu]

Fig. 3

*Hynobius* sp.: Matsui and Matsui, 1980, p. 104, figs. 1–4.

**Holotype.**—OMNH Am 9151, an adult male from Ochikura Swamp (137° 52'E, 36° 44'N, alt. 800 m) on the eastern slope of Mt. Shiro-uma-dake in Hakuba-mura, Minami-Azumi-gun of Nagano Prefecture, collected by Masafumi Matsui on 27 April 1983.

**Paratypes.**—OMNH Am 9152–9158, 6 males and 1 female, NSMT-H-04004–04010, 6 males and 1 female, with the same data as the holotype.

**Diagnosis.**—A member of the *nebulosus-lichenatus* complex: breeding in very slowly flowing water; adapted to cool climate; most similar to *H. lichenatus* in appearance, especially in shallow vomerine teeth series, and breeding habits, but has electrophoretic differences, an unstriped egg sac envelope, a smaller number of vomerine teeth, a relatively short and high tail, and relatively short limbs and long trunk.

**Description and variation.**—The following description is based on the maximum number of 40 adult males and 15 adult females. Morphometric data are summarized in Table 5 together with those on the allied species, *H. lichenatus*, *H. takedai*, *H. abei*, *H. tokyoensis*, and *H. nebulosus*. Head moderately depressed, distinctly longer than broad. Males with relatively larger head both in length (HL) and width (HW) than in females, when each dimension is converted to percentage ratio to SVL ( $U_{39,15}=151.5$ ,  $p<0.01$ , and  $U_{39,15}=84.5$ ,  $p<0.001$ , respectively; Table 5). Females with relatively long trunk ( $U_{39,15}=153$ ,  $p<0.01$ ). Number of costal grooves including axillary groove 12 to 13 (Table 6), the modal number being 12 in both sexes. Limbs short and stout, and when adpressed, they do not overlap and are always separated by at least 0.5 folds. Separation is greater in females (median = 2 folds) than in males (median = 1; Table 7,  $\chi^2=17.1$ ,  $dF=2$ ,  $p<0.001$ ). Tail vertically oval at base, gradually flattening to tip, which is

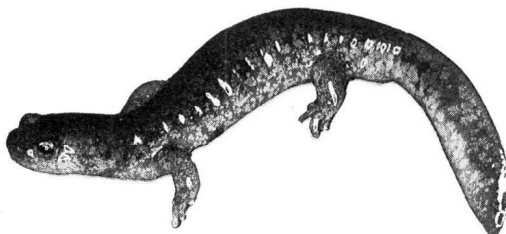


FIG. 3. A male paratype of *Hynobius hidamontanus* (OMNH Am 9156).

obtusely pointed. Posterior half of tail keeled, moderately above and weakly below. The upper keel very weakly originating at the position opposite to the posterior end of vent. No development of tail fin even in breeding males. Statistically significant sexual dimorphism in tail shape: males with relatively longer ( $U_{37,15}=34$ ,  $p<0.001$ ) and higher ( $U_{39,15}=151.5$ ,  $p<0.01$ ) tail than in females. Fifth toe always absent, and the 5th toe was not even rudimentary in any of the observed samples. Vomerine teeth in two small, obliquely arched series, nearly touching at midline, and usually forming a very shallow "V". Combined series distinctly wider than long (VTW/VTL 1.43–3.12 in males and 1.39–2.57 in females). A significant sexual difference in the number of vomerine teeth (Table 8,  $t=2.79$ ,  $dF=44$ ,  $p<0.01$ ). Length of vomerine teeth series (VTL) smaller in males than in females ( $t=2.77$ ,  $dF=35$ ,  $p<0.01$ ), but the width (VTW) does not differ ( $t=0.48$ ,  $dF=35$ ,  $p>0.5$ ). Shape of vomerine teeth series sexually different as shown by comparison of the ratio of VTW to VTL ( $U_{22,15}=75.5$ ,  $p<0.001$ ).

**Color in life.**—Dorsum hessian brown (dark brown), very finely dotted with small silvery and brassy speckles. These speckles tend to fuse on tail. Sides lighter, with many silvery flecks, excepting around costal grooves. The development of silvery flecks far stronger in females than in males. Underside lighter than dorsum, densely covered with silvery flecks.

**Color in preservative.**—Dorsal coloration, especially silvery speckles tend to fade. Otherwise no obvious change in color or pattern occurs, and silvery flecks on under-surface remain quite evident.

**Measurements and counts of the holotype.**—An adult male weighing 3.4 g (live) and with the following measurements (mm; in preservative): HW 9.0, HL 10.8, head depth at posterior angle of jaw 3.5, eyelid length 2.4, anterior rim of orbit to snout 2.7, horizontal orbit diameter 2.1, interorbital distance 3.1, snout to insertion of



TABLE 5. Comparisons of measurement (means  $\pm$  2SE, followed by ranges in parenthesis, in mm) and percentage ratios of each character dimension to SVL (medians, followed by ranges in parenthesis) in the six forms of the *Hynobius nebulosus-lichenatus* complex.

Form	Sex	N	SVL	N	HL/SVL	N	TRL/SVL	N	TAL/SVL	N	HW/SVL	N	MTAH/SVL
<i>H. hidamontanus</i>	M	39	47.7 $\pm$ 1.0 (41.1–54.4)	39	24.1 (21.6–26.7)	39	75.9 (73.3–78.4)	37	75.7 (68.4–85.6)	39	19.6 (17.7–21.1)	37	11.9 (9.4–15.1)
	F	15	51.3 $\pm$ 1.2*	15	23.2*	15	76.8*	15	69.7*	15	18.6*	15	9.6*
<i>H. lichenatus</i> (Aomori)	M	20	47.0–54.4	20	20.6–25.0	20	74.9–79.4	20	93.3*	20	19.8 (16.8–19.4)	20	11.2*
(Tochigi)	M	24	55.9 $\pm$ 1.5*	24	24.6	24	75.4	20	87.4*	24	18.7*	22	11.2*
	F	9	58.3 $\pm$ 1.1**	9	23.9	9	76.1	9	79.7**	9	18.9 (17.1–21.6)	9	8.7**
<i>H. takedai</i>	M	31	56.8 $\pm$ 1.7*	31	24.0	31	76.0	28	80.5*	30	20.2*	29	13.5*
	F	7	57.8 $\pm$ 1.8**	7	23.0	7	77.0	7	64.7**	7	18.7 (17.8–22.7)	6	10.3
<i>H. abei</i>	M	24	57.8 $\pm$ 2.1*	24	26.1*	24	74.0*	24	73.7	24	21.5*	24	17.1*
	F	34	55.0 $\pm$ 1.2**	34	25.4**	34	74.6**	33	64.5**	34	20.1**	31	12.8**
<i>H. tokyoensis</i>	M	21	56.7 $\pm$ 2.3*	21	22.6*	21	77.4*	21	76.5	21	18.2*	21	11.6
	F	6	63.9 $\pm$ 4.0**	6	23.5	6	76.6	6	72.8	6	16.7**	6	10.0
<i>H. nebulosus</i> (Shiga)	M	14	48.3 $\pm$ 2.9	14	24.4	14	75.6	13	86.9*	14	19.3 (16.4–17.5)	13	14.6*
	F	12	50.4 $\pm$ 2.1	12	23.0	12	77.0	12	70.8	12	18.3 (18.3–20.9)	12	11.4**
(Nagasaki)	M	32	59.2 $\pm$ 1.6*	32	23.5	32	76.5	23	69.7*	32	18.9*	32	12.4
			(50.9–66.9)		(21.0–25.7)		(74.3–79.0)		(64.0–80.4)		(17.2–20.4)		(9.5–14.5)

SVL=snout-vent length; HL=head length; TRL=tail length; TAL=tail length; HW=head width; MTAH=maximum tail height.

\* significantly different from male *H. hidamontanus* at  $p < 0.05$ .

\*\* significantly different from female *H. hidamontanus* at  $p < 0.05$ .

TABLE 6. Variation in the number of costal grooves, including the axillary groove, in the six forms of the *Hynobius nebulosus-lichenatus* complex.

Form	Sex	N	Number of costal grooves			
			11	12	13	14
<i>H. hidamontanus</i>	M	40		35	5	
	F	15		12	3	
<i>H. lichenatus</i> (Aomori) (Tochigi)	M	20		17	3	
	M	24	4	18	2	
	F	9	1	8		
<i>H. takedai</i>	M	31	1	26	4	
	F	7		4	3	
<i>H. abei</i>	M	24	2	22		
	F	34		30	4	
<i>H. tokyoensis</i> *	M	21		3	17	1
	F	6		2	4	
<i>H. nebulosus</i> (Shiga)* ** (Nagasaki)*	M	14			14	
	F	12			11	1
	M	32		3	27	2

\* significantly different from male *H. hidamontanus* at  $p<0.05$ .  
\*\* significantly different from female *H. hidamontanus* at  $p<0.05$ .

forelimb 15.7, distance separating internal nares 2.7, distance separating external nares 2.7, SVL 46.0, axilla to groin 23.4, TAL 38.3, tail width at base 4.8, tail height at posterior angle of cloaca 4.7, tail height at middle 6.1, MTAH 6.2, axilla to tip of outstretched forelimb 13.1, groin to tip of outstretched hindlimb 14.6, VTW 2.45, VTL 0.81. The holotype has 58

upper jaw teeth, 62 lower jaw teeth, 21 vomerine teeth, 12 costal grooves between axilla and groin including axillary groove, addressed limbs separated by 1.5 costal folds, and 4 digits on both hindlimbs.

*Etymology.*—The specific name “*hidamontanus*” refers to the so-called North Japan Alps, the Hida Mountains, where this species was found.

*Range.*—Known so far only from Central Japan, the montane region of the northwestern part of Nagano Prefecture, on the Hida Mountains (Fig. 4). Hakuba-mura: Ochikura swamp.

*Morphological comparisons.*—In SVL, *H. hidamontanus* was not different from *H. nebulosus* from Shiga in either sex (Table 5), but was smaller than the other species and/or populations in both sexes. By comparing percentage ratios of each character dimension relative to SVL, the following significant differences ( $p<0.05$ ) in body shape were detected.

In males, compared with *H. lichenatus* from Aomori and Tochigi, *H. hidamontanus* had shorter or broader head, longer trunk, and shorter but higher tail. *Hynobius hidamontanus* had longer but narrower head and shorter and lower tail than *H. takedai*. *Hynobius abei* differed from *H. hidamontanus* in longer and broader head, shorter trunk, and much higher tail. *Hynobius tokyoensis* had smaller head, longer trunk, and longer tail than male *H. hidamontanus*. *Hynobius nebulosus* from Shiga

TABLE 7. Variation in the number of costal folds between addressed limbs in the six forms of the *Hynobius nebulosus-lichenatus* complex.

Form	Sex	N	Overlap of addressed limbs shown by number of costal folds											
			-3	-2.5	-2	-1.5	-1	-0.5	0	0.5	1	1.5	2	2.5
<i>H. hidamontanus</i>	M	39		1	4	14	18	2						
	F	15	1	1	7	6								
<i>H. lichenatus</i> (Aomori)* (Tochigi)* **	M	20									6	10	2	2
	M	24					3		2	3	9	6	1	
	F	9					1	1	6	1				
<i>H. takedai</i> *	M	30			1	6	4	6	5	3	5			
	F	7		1	4	2								
<i>H. abei</i>	M	24	1		4	6	12	1						
	F	34	3	8	16	5	1	1						
<i>H. tokyoensis</i> *	M	21			1	2	9	6	2		1			
	F	6		1	3	1	1							
<i>H. nebulosus</i> (Shiga)* * (Nagasaki)*	M	14			3		4	2	4	1				
	F	12			5	6	1							
	M	32	4	6	9	5	4	3	1					

\* significantly different from male *H. hidamontanus* at  $p<0.05$ .  
\*\* significantly different from female *H. hidamontanus* at  $p<0.05$ .

TABLE 8. Comparisons of number of vomerine teeth (mean $\pm$ 2SE) and shape of vomerine teeth series (median) in the six forms of the *Hynobius nebulosus-lichenatus* complex. Figures in parenthesis indicate variation range.

Form	Sex	N	Vomerine teeth number	N	VTW/VTL
<i>H. hidamontanus</i>	M	31	25.4 $\pm$ 1.0 (20–29)	22	2.39 (1.43–3.12)
	F	15	27.7 $\pm$ 1.2* (25–32)	15	2.03* (1.39–2.57)
<i>H. lichenatus</i> (Aomori)	M	20	30.1 $\pm$ 1.4* (25–35)	20	2.58 (1.59–2.93)
	(Tochigi)	M	24	19	2.26 (1.66–2.68)
<i>H. takedai</i>	F	9	35.3 $\pm$ 1.8** (32–41)	9	2.29 (1.60–3.08)
	M	30	37.1 $\pm$ 2.2* (28–54)	21	1.76* (1.19–2.66)
<i>H. abei</i>	F	7	35.6 $\pm$ 4.7** (27–44)	7	1.68** (1.38–2.38)
	M	24	36.3 $\pm$ 1.9* (28–46)	20	1.37* (1.21–1.62)
<i>H. tokyoensis</i>	F	34	36.0 $\pm$ 1.1** (31–43)	19	1.46** (1.25–1.69)
	M	21	31.2 $\pm$ 1.6* (26–41)	21	1.69* (1.39–2.31)
<i>H. nebulosus</i> (Shiga)	F	6	38.3 $\pm$ 4.6** (34–46)	6	1.54* (1.39–1.98)
	M	14	41.9 $\pm$ 2.7* (31–50)	14	1.16* (0.91–1.41)
(Nagasaki)	F	12	42.3 $\pm$ 2.6** (36–50)	12	1.19** (0.98–1.43)
	M	32	49.4 $\pm$ 2.6* (34–67)	32	1.04* (0.85–1.32)

VTW=width of vomerine teeth series; VTL=length of vomerine teeth series.

\* significantly different from male *H. hidamontanus* at  $p < 0.05$ .

\*\* significantly different from female *H. hidamontanus* at  $p < 0.05$ .

differed from *H. hidamontanus* in having longer and higher tail, but the same species from Nagasaki had conversely shorter tail than *H. hidamontanus*. In females, limited number in some samples prohibited detailed comparisons, but tendencies similar to those found in males were sometimes evident. *Hynobius hidamontanus* had shorter but higher tail than *H. lichenatus*, but had longer tail than *H. takedai*. Compared with *H. abei*, *H. hidamontanus* had smaller head, longer trunk, and longer but lower tail. From *H. tokyoensis* and *H. nebulosus*, *H. hidamontanus* differed only in having broader head and higher tail, respectively.

Both sexes of *H. hidamontanus* had modally 12 costal grooves, and in this respect, were similar to *H. lichenatus*, *H. takedai*, and *H. abei*. This modal value was significantly smaller than that possessed by male *H. tokyoensis* and both sexes of *H. nebulosus* (Table 6). The insignificant difference found between females of

*H. hidamontanus* (mode=12 grooves) and *H. tokyoensis* (mode=13) in the number of costal grooves may be due to small sample size of the latter species. Males of *H. hidamontanus* somewhat differed from males of the other species, except for *H. abei*, in the degree of limb overlap (Table 7). In male *H. hidamontanus*, limbs were more widely separated than in males of *H. lichenatus*, *H. takedai*, *H. tokyoensis*, and *H. nebulosus* from Shiga. The last species from Nagasaki, however, had limbs even more widely separated than *H. hidamontanus*. In females, *H. hidamontanus* showed wider separation of limbs than *H. lichenatus*, but had insignificant differences from the remaining species.

In both sexes of *H. hidamontanus*, the number of vomerine teeth was significantly smaller than in all other species (Table 8). The shape of the vomerine teeth series as expressed by the ratio of width and length also differed from all other species, except for *H. lichenatus*. Both sexes of

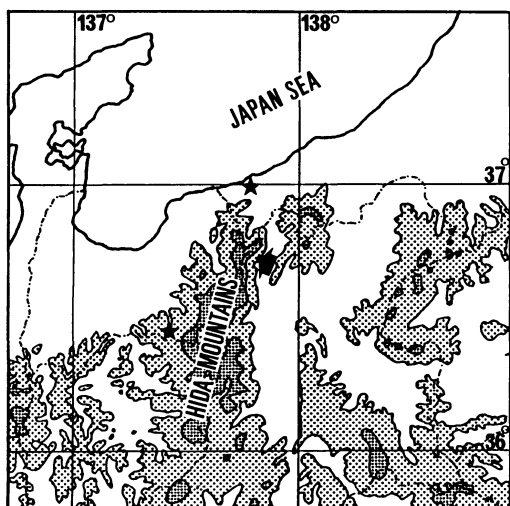


FIG. 4. A map of northern Central Honshu, Japan, showing the type locality of *Hynobius hidamontanus* (black arrow) and localities for the salamander reported by Nambu (1983), whose taxonomic status is uncertain (black stars). Coarsely dotted area = >1000 m alt. Finely dotted area = >2000 m alt.

the latter species and *H. hidamontanus* had much shallower vomerine teeth series than other species.

**Isozymes.**—No electromorphs unique to *H. hidamontanus* have hitherto been detected, but the following number of fixed allelic differences are evident: one from *H. lichenatus* (S-Mdh-A), two from *H. nigrescens* (Pgdh-A, S-Me-A), three from *H. takedai* (M-Mdh-A, Pgm-C, Pgdh-A), eight from *H. abei* (S-Aat-A, M-Mdh-A, S-Mdh-A, S-Sod-A, Pgm-A, Pgm-C, Est-2, Ldh-A), three from *H. tokyoensis* (S-Aat-A, Pgm-C, Ldh-A), and two from *H. nebulosus* (Pgdh-A, Est-1).

**Fecundity and natural history.**—Clutch size and morphology of egg sac were described by Matsui and Matsui (1980), and additional data are now available (data largely from Kakegawa in litt.). The clutch size varies from 30 to 76 and the mean is  $51.8 \pm 2.6$  (2SE,  $N=59$ ). This value is intermediate between those reported for *H. lichenatus* (mean =  $40.1 \pm 2.3$  in southern populations) and *H. abei* (mean =  $71.3 \pm 6.9$ ) (Matsui and Miyazaki, 1984). The newly accumulated data for northern populations of *H. lichenatus* from near its type locality also indicate the tendency toward slightly smaller clutch size of this species than *H. hidamontanus* (Aomori: mean =  $47.1 \pm 9.3$ ,  $N=12$ ; Hirosaki: mean =  $39.3 \pm 7.7$ ,  $N=16$ ).

The mean diameter of each ten ova just oviposited by four females ranged from 2.57–3.10 (mean =  $2.77 \pm 0.24$  (SD)) mm. The animal pole is dark brown and the vegetal pole is grayish brown in color. The surface of the egg sac completely lacks the strong longitudinal striations found in *H. lichenatus* and *H. abei* (see Fig. 4 in Matsui and Matsui, 1980). The natural history of *H. hidamontanus* is very incompletely known; breeding occurs from late April to early May, and in some larvae metamorphosis occurs in the year following oviposition.

**Protection.**—The population from Hakubamura has been tentatively referred to *H. lichenatus*, and is now protected by the Hakubamura Village Board of Education.

#### DISCUSSION

Clearly, the analysis of isozyme variation is quite useful for outlining species among Japanese salamanders, since populations of each hitherto named species were grouped into one unit in a UPGMA phenogram. On the other hand, the genetically assessed relationships as revealed by the phenogram are largely not in favor of the currently popular phylogenetic idea from morphology and/or ecology (Sato, 1943).

Sato (1943: 24, 500) proposed to split Japanese species of the genus *Hynobius* into mountain stream types (lotic breeders) and lowland still-water types (lentic breeders), and further subdivided the latter type into two groups—the *nebulosus* group adapted to warm climate (*H. nebulosus*, *H. tokyoensis*, *H. dunni*, *H. tsuensis*), and the *lichenatus* group adapted to cool climate (*H. lichenatus*, *H. abei*, *H. nigrescens*, *H. sadoensis*). This classification is ecologically incorrect, because *H. tsuensis*, *H. lichenatus*, and even some populations of *H. nebulosus* are strictly not lentic, but lotic breeders (Matsui, unpubl.). They are, however, morphologically quite uniform when compared with the primarily lotic *H. naevius* group, and in this respect, part of Sato's idea can be advocated. Regarding the subdivision of 'lentic' breeders, however, the results of isozymic analysis, as discussed below, are in disaccord with Sato's idea, as is the available karyological evidence (Seto et al., 1986). Therefore, I combined all the 'primarily' lentic-breeding species into the *nebulosus-lichenatus* complex in the present work.

Considerable genetic variation was found among populations of *H. lichenatus*. This result partly coincides with the high morphological variation in this species (Hasumi and

Iwasawa, 1987), and suggests the presence of several cryptic taxa in this wide-ranging species. To examine this possibility, I am currently conducting a larger degree of variation analysis both morphologically and genetically. *H. lichenatus* is largely sympatric with *H. nigrescens* in its range, and I have examined two sympatric populations (Minakami and Shiobara) of these two species in this work. The result indicated a clear genetic isolation between these species ( $D=0.422$  and  $0.483$ ), and no evidence of introgression or hybridization was detected.

Among six already named species treated here, *H. nigrescens* is morphologically most conspicuous both in adults and egg sacs. The species has been placed in the *lichenatus* group and regarded as a close relative of *H. lichenatus* and *H. abei* (Sato, 1943: 119). The result of isozyme analysis, however, indicates that *H. nigrescens* is remote from these two species (mean  $D=0.358$  and  $0.590$ , respectively), but is close (mean  $D=0.228$ ) to *H. nebulosus* of the *nebulosus* group, which is morphologically of a general type and lacks any specializations.

*Hynobius takedai* was originally identified as *H. abei* (Miyazaki, 1977), and later described as a close relative of *H. lichenatus* (Matsui and Miyazaki, 1984). The result of isozyme variation analysis, however, was in disaccord with these assumptions and the species is genetically closer to *H. nigrescens* (mean  $D=0.234$ ) than to either *H. lichenatus* (mean  $D=0.573$ ) or *H. abei* (mean  $D=0.919$ ).

In assigning a salamander from lowland of Toyama Pref. to *H. takedai*, Nambu (1986) noted the difficulty in identifying salamanders on a morphological basis and adopted several additional measurements besides the 'routine' ones. Even so, he failed to show any concrete basis on which his identification was made. Indeed, identification of salamanders from morphology and/or ecology alone can be made at present only within certain limits.

The present result of the examination of the isozyme variation clearly indicates a close genetic similarity of the salamander population from lowland of Toyama to *H. takedai* ( $D=0.026$ ), and, in a strict sense, first clarifies the taxonomic position of that population.

Genetically, the most conspicuous species with a markedly great genetic distance from other species (mean  $D=0.590$ ) is not morphologically unique *H. nigrescens*, but *H. abei*. The species has been noted for its isolated distribution and early breeding, and the genetic

evidence further strengthens its unique position among the *nebulosus-lichenatus* complex.

*Hynobius tokyoensis* is currently often regarded as a subspecies of *H. nebulosus* (Nakamura and Uéno, 1963), and their close relationship has never been challenged. The present result of the isozyme analysis, however, is again in disaccord with the current taxonomic idea. *Hynobius tokyoensis* is closer to *H. lichenatus* (mean  $D=0.223$ ) than to *H. nebulosus* (mean  $D=0.447$ ), which in turn is closer to *H. nigrescens* (mean  $D=0.228$ ). However, notwithstanding its wide distribution, only one population (Shiga) of *H. nebulosus* was examined in the present study. Consequently, whether the result obtained for this population holds or not needs to be clarified in future studies.

The newly described *H. hidamontanus* has been regarded as a close relative of *H. lichenatus* (see Matsui and Matsui, 1980), but the isozymic evidence indicates that the species is closer to *H. nigrescens* (mean  $D=0.279$ ) and *H. nebulosus* ( $D=0.298$ ) than to *H. lichenatus* (mean  $D=0.363$ ). This result again contradicts splitting these salamanders into the *lichenatus* and *nebulosus* groups. The population from O-umi, Niigata, was found to be genetically closest to *H. hidamontanus* ( $D=0.195$ ). Several diagnostic characteristics such as non-striped egg sacs, primary absence of the fifth toe on hindlimb, and small number of vomerine teeth reported for the salamanders from O-umi (Nambu, 1983) strongly suggest its conspecific status with *H. hidamontanus*. The salamander from Ooyama, Toyama, also reported by Nambu (1983), again shares the diagnostic characteristics with *H. hidamontanus*. These two populations, as well as *H. hidamontanus*, are distributed in a region surrounding the northern half of the Hida Mountains (Fig. 4), and are geographically isolated from *H. lichenatus* by the western edge of the Fossa Magna (Itoigawa-Shizuoka tectonic line). Probably because of the topography of their habitat, information on the distribution of salamanders from this region is meagre, and more detailed taxonomic and biogeographic studies are required for the better understanding of phylogenetic relationships among salamanders of the *nebulosus-lichenatus* complex around this region.

As shown above, isozymic divergence seems to be not coupled with currently recognized morphological and/or ecological divergence in the *nebulosus-lichenatus* complex of the genus

*Hynobius*. This discordance is not astonishing, partly because morphological and ecological data have so far never been adequately analyzed, and partly because 'intermediate' forms between the *nebulosus* and *lichenatus* groups of Sato (1943), such as *H. takedai* and *H. hidamontanus*, have recently been discovered from geographically 'intermediate' areas. It is, of course, also to be noted that the systematic discrepancy shown above may be due partly to sampling biases, and partly to the method of genetic analysis employed. Future studies with a larger number of samples from representative localities of species ranges, and the use of various analytical methods both for the genetic and morphological and/or ecological data would clarify the gap here indicated.

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要旨 本州東部産カスミサンショウウオオートウ  
ホクサンショウウオ複合群におけるアイ  
ソザイムの変異と1新種の記載

松井 正文

近畿地方以北に分布するカスミサンショウウオオートウホクサンショウウオ複合群244個体のアイソザイムについて、デンブングル電気泳動法を用い、17遺伝子座における変異を調べた。これまでに命名されていた6種(トウホク、クロ、ホクリク、アベ、トウキョウ、カスミ)は比較的小さなNeiの遺伝的距離(2種個体群間距離の平均の最小値=0.22)によって、たがいに明瞭に区別された。しかし、これらの種間にみられた遺伝的な関係と、これまで認められてきた系統関係との間には大きな不一致があった。白馬村産のサンショウウオ個体群は、他のすで

に命名されている種と比較した場合に、独立種として認めるに足るほどの大きな遺伝的相違を示した。この結果にもとづいて、白馬村産の個体群を新種ハクバサンショウウオとして記載する。この種は中部地方飛騨山脈に含まれる、長野県の山地帯に分布する。カスミサンショウウオオートウホクサンショウウオ複合群に属し、鋤骨歯列が浅いこと、鋤骨歯数が少ないこと、トウホクとカスミの中間的体型をもつこと、卵囊外被に縦条を欠くこと、独特のアイソザイム泳動パターンをもつこと、で特徴づけられる。

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